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STAIN TECHNOLOGY

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STAIN TECHNOLOGY

VOLUME III JANUARY, 1928 NUMBER 1

THE HISTORY OF STAINING

THE PIONEERS IN STAINING

H. J. CONN, *Geneva, N. Y.*

PRIMITIVE METHODS OF STAINING COMPARED WITH MODERN

There is nothing more difficult than to establish the earliest date at which some very common procedure was first used. Such dates, apparently fixed, have a way of becoming unfixed as the history of the subject is further studied.

Thus it was once thought that Gerlach (1858)[1] had introduced the use of dyes in microscopic work. But Gierke (1884) pointed out that, in spite of the credit which Gerlach deserved in the matter, he was not the first to employ dyes for this purpose. The earliest reference given by Gierke is to the work of Göppert and Cohn (1849); thus he in turn overlooked the work of Ehrenberg (1838) which has since been cited some times as probably the first microscopic use of dyes.

Holzner (1884) ascribes the earliest use of stains in histological work to Sarrabat (1733); or if there is question as to whether he actually made histological application of the dyes, claims that the credit goes to Reichel (1758). Both of these investigators used colored solutions to observe the rise of fluids in sticks dipped into them; but from Holzner's quotations it is apparent that their observations were macroscopic rather than microscopic. It is a muted question, therefore, whether this can be called an histological employment of dyes.

Whether or not it is correct to ascribe priority to either Sarrabat or Reichel, it is plain that we must go back earlier than the 19th century to find the first use of dyes in histology. Thus G. M. Smith (1915) shows that Hill (1770) had employed dyes in studying the microscopic structure of timber, and that, beyond question, his work was actually histological in nature. His methods, in fact, were more like modern histology than the subsequent work of Ehrenberg. This may or may not settle the matter. The publication of scientific work earlier than 1800 was mostly in sources difficult to obtain today; and there is no knowing what future students will unearth.

[1]For literature cited see bibliography at end of paper.

Nevertheless, even tho the earliest date of microscopic staining has thus been pushed back nearly a century before the time of Gerlach, the fact remains, as acknowledged by Gierke, that Gerlach was actually the founder of staining technic, even tho not the discoverer of the use of stains. Before his day staining was very unlike that of modern histology. To translate a passage from Gierke:

"It is very profitable for us moderns to cast a glance occasionally on the condition of histology before 1858. And when we recognize what our teachers and leaders accomplished without present-day methods, we will do well to bow our heads deeply before them. If we, accustomed as we are today to ascribe to staining reactions such a large share of the results of modern investigation, would only realize how much the earlier histological science, without them, had established; if we would study carefully the manuals on microscopic anatomy written before 1858; then the thought would become strong within us: 'The keen eyesight of the investigator and his ingenuity should be able to do something still greater in histology.' And I say, God be praised that it is so! For otherwise we would, in fact, degenerate into manual laborers and our results would be wholly dependent upon good stains. And this would be all the more deplorable because—the unwelcome fact must be admitted—our previously so highly prized methods were never employed in a scientific way, and can never be so employed. I will come back to this matter later, but will at this point assert that we are very much in the dark in regard to the operation of staining reagents. We are probably correct if we assume that in staining we are dealing with a chemical process; but that is as far as we can go."

This quotation is interesting, not only in pointing out the difficulties in histological technic before the days of staining, but also in hinting at the crudeness of staining methods in the eighties. It is still true that in getting a certain result in staining, the ingenuity of the technician is more important than the quality of the stain; but who today would have the temerity to hint, as apparently implied by Gierke, that the skill and keen eyesight of the investigator could make him independent of good stains? With the modern complicated procedures of double and triple staining, even a remarkably skilled technician can hardly expect good results with undependable stains.

An idea of the simplicity of early staining procedures can be obtained from the following translation from Hartig (1854 c, p. 895):

"The procedure for the investigation was essentially as follows: . . . The onions, having sprouted in the air of a warm room and grown to 2–4 inches in length, were dissected, the sprouts loosened at their base and boiled 12 to 16 hours. Softened in this way, the epidermis, with the outermost layers of cells and the stomata, could easily be pulled off in large pieces with forceps and dissecting needle, and spread out on a slide under water; whereupon after the drying of the edges

of the slide, the dye solution was added, and with frequent renewing held until the cell nuclei had a color satisfactory for observation; whereupon, if one wishes to preserve satisfactory preparations, the superfluous dye should be washed off with distilled water and a few drops of dilute zinc chloride solution."

The extreme casualness of this reference to staining can best be appreciated if it is understood that the article seems to contain no previous reference to any dye solution; and it is not until a few pages later (p. 899) that the following statement makes it apparent that the writer was using carmin:

"The division of the cell nucleus shows most distinctly in *Allium Porrum*, in which the division line can often be recognized even before the entrance of the cell nucleus by a zone colored less dark with carmin, and often thru a slight notching at this point."

Apparently there was no great variety among dyestuffs in those days nor was it considerd very necessary to mention what strength solutions were used or how they were applied!

During the following quarter century stains came to be used more and more in microscopy; until in 1883 Gierke remarks:

"If a layman today steps into the room of an histologist he marvels most at the array of colors in the quiet work-room. It is not the strange apparatus, the microscope and the microtome with its dangerous-looking knives which first catches the visitor's eye; it becomes riveted to the many gay-colored objects. He had pictured the microscopic laboratory as bare and monotonous, for in his opinion the scientific material therein becomes visible only upon hundredfold magnification. Instead, the brightly lighted room shines with the splendor of gay colors. In cupboards and on the tables are all sorts of flasks, with large and small dishes containing colored substances and sparkling liquids, while everywhere on the tables are little glass plates to which objects, shining with all colors of the rainbow, are fastened. Can those be the microscopic preparations, supposedly so insignificant looking to the naked eye?"

If such a picture were true in 1883, how much more so now, forty-five years later! To watch the development of the staining technic it is necessary to follow the work of the early histologists.

The Early Microscopists

The two countries in which the microscope was at all commonly used before the days of modern histological technic were England and Germany. Its use, however, was for distinctly different purposes in each of these two countries. The German microscopist was a scientist, much more interested in learning facts about nature than in making fine microscopic preparations; to him the microscope was merely a tool, and if he was able to get the results he desired,

he did not care to take time to perfect his technic. The English microscopist, on the other hand, was usually an amateur, interested in the microscope for the sake of the marvels it could reveal and the beautiful preparations which he could have on hand to display to others; so by him any improvement in technic was gladly welcomed. As a result the early refinements in microscopic technic came from England rather than Germany. The English microscopists seem to have originated the ivory "slider," the predecessor of our microscopic slide; and the first microtomes were of English manufacture. In fact the early German microscopists seemed to have scorned the laboratory worker who could not get good results with free-hand section. Later, of course, as the details of tissue and cell structure began to be appreciated, the Germans realized the necessity of better technic; and from that time on the refinements in technic originated largely in Germany.

As the development of the microscope and of microscopic technic from that time on centered around Germany, it is natural that the early work of the English microscopists was forgotten. G. M. Smith (1915) seems to deserve the credit of bringing it to the attention of Americans. Hill's work, referred to above, on "The construction of timber, explained by the microscope" is especially interesting. The following quotation from Smith (1915) makes the character of the work of this early investigator quite apparent.

"Hill did not rely on any one method, but studied the structure of stems in many ways. He used a more elaborate method of maceration than Martin, and was the first to use maceration in the study of wood, sinking a loose wicker basket containing the sticks he wished to study into a stream until the tissues were well softened. This identical method, which was rediscovered by the younger Moldenhawer in 1812, is considered by Sachs as one of the great steps in the progress of phytotomy. Hill also makes the first mention of a method of preservation of material for further study. The practice of dropping the macerated pieces of wood into a solution of alum and then transferring them to spirits of wine, after thoroughly drying, resembles in a very crude manner our modern method of fixing and hardening. The reason for this is seen in his statement that 'Nothing but spirit of wine can preserve these tender bodies, and, till I found this method of hardening them first, the liquor often destroyed them. . . .'

"Hill is undoubtedly the first to have used staining as an aid in the study of microscopical anatomy of plants. He prepared an alcoholic tincture of cochineal, in which, after it had been filtered, he placed the stems of plants for a while, discarding that portion which had been immersed in the fluid when he made his sections. Another method of staining used was even more advanced since it involved a mordanting of the tissues before developing the color. A solution of sugar of lead was prepared, filtered, and put in a tea-cup

and the sticks to be studied were allowed to remain in this fluid for two days. An essential part of this operation was the 'whelming' of the tea-cup with a wineglass to prevent the drying of the material. While the tissue was soaking in the lead solution he prepared a solution of quick lime and orpiment in water and then transferred the material from the tea-cup to the second solution for two days. When the sticks were first placed in the second solution they were colorless, but in a short time they became deep brown. By means of this staining he was able to demonstrate the existence of structures invisible in the uncolored material. A third method, which was an injection rather than a staining process, was by careful drying of the wood and boiling it in green sealing wax. By this procedure the vessels became thoroughly impregnated with the green sealing wax and the 'split pieces resemble striped satins, in a way scarce to be credited.' "

Writers concerning the history of the microscope generally describe the 18th century as being barren of developments as compared with the 17th; and the work of Hill stands out all the more prominently on that account. No further reference to the use of carmin or other dyes in this sort of work seems to be on record until that of Ehrenberg (1838). This investigator is especially noted as one of the earliest students of infusoria, especially the bacteria. He insisted that the infusoria had internal organs; and that by the aid of colored organic matter it is possible to recognize alimentary organs in almost all of these organisms. He claimed that no absorption of colored matter takes place thru the surface of the animalcule; but that after remaining some time in colored water the body of the organism is still uncolored, while the "stomachs" are full of colored matter. These results, he declared, can be obtained merely by "serving" indigo or other colored vegetable substances to the organisms; whereupon one can shortly see the animalcules "fill themselves" with these substances. Thus he thought he recognized several stomachs, and named the group of organisms: Polygastrica.

It is plain today how Ehrenberg came to go astray in this matter. Differential staining was not then recognized; and it was difficult for him to understand how the carmin could color the inner portions of the cells without staining the outer parts.

Ehrenberg's work was not histology, and his use of a dye was not for the purposes for which they are employed by modern microscopists. A decade later, however, Göppert and Cohn (1849) used carmin and madder with the very evident intention of making the cell contents more visible. They employed these dyes rather incidentally in the course of a study on the rotation of the cell contents of *Nitella flexilis*.

Apparently the next employment of any dye in microscopic work was by Corti (1851) in studying the epithelial lining of the cochlea. This investigator had very plainly developed a skilful technic of dissection; but altho he realized the value of stains, his staining methods were quite crude. The contrast between his detailed description of his technic of dissection and his casual reference to staining is best brought out by one of the notes which he appends to his article, this note referring to a paragraph of his paper in which he describes the size and appearance of the cells of this epithelium. This note seems to be the only reference in his paper to the use of any dye. A translation of it follows:

"The epithelial layer which lines the cavity of the cochlea cannot be seen in its natural state except in very fresh preparations and by employing a moderately concentrated solution of sugar. The study of the histological elements of the cochlea requires, if possible, more caution than the study of the retina. I think, therefore, it will not be amiss to add the method of preparation which I have found the most favorable for the purpose. In general the essential condition in making such preparations is that the animals must be dissected immediately after death and while they are still warm. If the animals are dead only six or eight hours, it may well be impossible to recognize some of the objects.

"One may detach quite large sheets of an epithelial layer which one has preserved several days in a saturated solution of corrosive sublimate. This seems to be due to the fact that the cell walls have assumed a tougher consistency and are attached to each other more strongly than in the natural state. With the same technic one may easily see the epithelial layer which lines the bony spiral lamella, rendering the latter transparent by means of acid. A very small quantity of chromic acid has almost the same action as corrosive sublimate. A chromic acid solution somewhat concentrated, on the other hand, makes the epithelial cells contract in such a manner that one can hardly recognize them, even if one still sees in them some nuclei. If a piece of the epithelium is fixed in ether or alcohol long enough, the cells shrink a little but they preserve their shape quite intact. One can distinguish in them, just as well as in the fresh condition, the cell-wall, the nuclei, and the nucleoli, especially when treated with acetic acid.

"The epithelial cells, when in their fresh condition, are almost entirely destroyed by concentrated acetic acid; this acid, even tho diluted with considerable water, makes them so transparent that they can hardly be recognized. Nevertheless, after the cells have been treated with solutions of sublimate, of chromic acid, and of alcohol and ether at a high temperature, they are only very slightly sensitive to the action of acetic acid, even pure, which then renders them merely a little transparent. This fact is somewhat remarkable.

"To observe distinctly the epithelial layer and its elements it is useful to color it lightly with a solution composed of half water and half alcohol in which sugar and carmin in sufficient quantity have

been placed. In such a solution, especially in the absence of air, the epithelial layer is well preserved—better than in any other solution. The nuclei in particular become very distinct because they take a color deeper than the rest of the cells."

This use of carmin comes quite close to an histological application of the dye. Yet apparently the differential staining secured was accidental, and the writer did not attach much significance to it. The next investigator to employ stains, however, regarded such observations as quite significant. This was the botanist, Hartig, who was apparently unaware of Corti's work but who refers to that of Göppert and Cohn. Repeating their technic with carmin, he confirms their finding as to its affinity for the granules (chlorophyl) which he names "chlorogen." He states (1854a) that carmin is not the only colored substance which is taken up by chlorogen, mentioning gamboge, cinnibar, copper sulfate, litmus and ink. He is much more interested in explaining this action than any of his predecessors had been, as shown by the following translation from his paper:

"One can hardly refer to this as a chemical reaction nor as a simple coloring by penetration, for the slightest, scarcely perceptible admixture of this with cell-sap colors the chlorogen and nothing else, neither the cell wall nor the cell-sap with which it is surrounded. The staining takes place in a few seconds and proceeds to completion in a few minutes. Carmin, litmus, the juice of *Phytolacca decandra*, accumulate to such a degree inside the solid substance of the chlorogen that this finally becomes entirely opaque and appears black under the microscope. The whole phenomenon cannot be explained except by assuming an extremely rapid passage of the surrounding fluid thru the chlorogen bodies by means of which the dyestuff dissolved in this fluid remains behind and accumulates therein. In this property of chlorogen, to absorb and to condense the substances dissolved in the fluid, its physiological significance must be searched for, its function thus seeming to be on the boundary between assimilation and excretion."

It seems strange today to see how many theories were suggested to explain differential staining before the idea was conceived that different parts of the cell might have affinities for different kinds of dyes.

In two papers later the same year Hartig (1854 b, c) follows up this same line of thought. The first of these papers reports considerable investigation as to the ability of the various parts of the cells to take carmin, and in this way he obtains what he regards as strong evidence in support of his theory that the chlorophyl granules are absorptive organs. He also made the rather more important observation that the nucleus of a cell stains only after

it has been killed; and that albumin and gelatin are readily stained by carmin, while gums, mucous, etc., do not stain. At this time he predicts a great future for the staining method in microscopic work.

Two years later Osborne (1856) in apparent contradiction to Hartig, observed the coloring of the cell contents in plants grown in colored solutions—carmin, indigo, or vermilion. This paper has sometimes been cited as the first use of staining in microscopy.

Gerlach and His Followers

The papers just mentioned are all interesting from an historical point of view; yet none of them contributed a great deal to the development of technic, for they were little known at the time. The work of Gerlach (1858),on the other hand, had a great deal of influence. Altho, as pointed out by Gierke, Gerlach's scientific observations merely showed in the case of human histology the same facts that Hartig had already brought out in regard to plants, he managed as we would say today, to "sell" his methods to others, and he therefore came to be regarded as the first to use stains in histology.

Joseph von Gerlach (who lived from 1820 to 1896) was professor of anatomy and physiology at Erlangen. At least four years before the date of his paper cited above he began the use of carmin, first injecting capillaries with a transparent mixture of carmin, ammonia and gelatin. He discovered that the nuclei stained more deeply than the cells and intercellular substance. Interested by this fact he began staining sections of the central nervous system with a concentrated carmin solution, but did not get satisfactory results, for the elements were not sufficiently differentiated. By accident, however, he happened one day to leave a section of brain tissue overnight in a dilute carmin solution. The result was good differentiation; and he had made the discovery that previous solutions were too concentrated for good staining effects.

In another paper the same year Gerlach (1858 b) reports attempts to stain living animal tissue. This never succeeded; and thus he corroborated the results Hartig had already obtained in regard to plant tissue. Dead tissue, on the other hand, Gerlach found to withdraw almost all the color out of a dilute carmin solution and to absorb it in such a way that it appeared mostly in the nuclei and nuclear granules, less in the cytoplasm and least in the intercellular substance. The color thus absorbed could not be washed out. This, he concluded, indicated a specific attraction between the dye and the cellular elements, concerning the physiological cause of which he had then no suggestion to make.

The great importance of this work seems to have been that he controlled his methods sufficiently so that they could be carefully described and followed by others. Gerlach was a prominent teacher and anatomist, and was able to show others the importance of staining technic.

The next paper of importance in this line, nevertheless, was by an investigator, Maschke (1859), who apparently was not familiar with Gerlach's work. Maschke had seen Hartig's work and considered that the latter had introduced a very important principle in employing dyes for microscopic purposes. He did not agree with Hartig, however, in explaining the accumulation of color in the chlorophyl granules or nuclei. Maschke was an apothecary, not a biologist; and his thoughts naturally ran to chemical rather than physiological theories. The following is a translation of one of his most significant statements on the subject:

"We have thus, in the above, learned to recognize two groups of organic substances, one of which combines with dyes, while the other shows no such property under the microscope; both groups gain in significance from the fact that one contains the various members of the protein family, the other those of the cellulose family, and that almost all members of the latter family are sole constituents of plant substances."

He then goes on to say that future work on the affinity of proteins for dyes may serve to differentiate between the different members of this group, so that proteins may become as well known as carbohydrates. He predicts that dyes will become important reagents in organic chemistry. Then he continues:

"Not only in chemistry, but also in the anatomy of plants and animals, the dyes have a decided worth. By means of the anatomical reagents it is not possible to determine of what the organic structures consist, but rather to make visible to the eye the various layers and forms, either by separation or by coloring; by this means these reagents have the effect of increasing extraordinarily the clearness and sharpness of our microscopes. By the use of dye solutions we can now by means of differential staining or by the alternation of stained and unstained condition, often make relations visible in a surprising way which otherwise would be hidden to the eye; the result which Hartig recently accomplished in his study of the granular contents of plant cells is especially attributable to his carmin solution.

"And thus I offer this reagent to physiologists in warmest terms; staining solutions will, I am sure, become as indispensible in the future as iodine solutions and both will share the place of honor beside the microscope together with the dissecting knife."

Thus Maschke, at last, came quite close to explaining the different staining ability of the different cell structures in terms like those used today. These early histologists had not employed acid dyes;

and not until acid and basic dyes were used in comparison could a clear conception of differential staining be obtained.

Following 1860, staining methods began to take on a much more modern character. The value of dyes in histological work was now appreciated and histologists began employing all the dyes then known. Maschke (1859) had introduced indigo. The anilin dyes were just coming on the market and were first employed in histology by Beneke (1862). Logwood extract was employed by Waldeyer (1864) in a rather unsuccessful attempt to stain axis-cylinders; while a crystalline hematoxylin was used by Böhmer (1865) in a formula that remained for a long time one of the most important histological staining solutions.

These and other developments later than 1860 will be taken up in other articles.

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STAIN SOLUBILITIES

Part III

W. C. Holmes

Contribution No. 145 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, Washington, D. C.

Further solubility data on dyes are recorded in the following table. The methods employed were those previously outlined[1]. In each instance the temperature was 26°C, and the result has been expressed in terms of grams of dye per 100 cubic centimeters of saturated solution.

The methylene violet listed is the azine dye and should not be confused with the thiazine dye, methylene violet (Bernthsen), which is found in polychromed methylene blues and is a constituent of MacNeal's tetrachrome stain.

Attempts to determine the aqueous solubilities of Biebrich scarlet, Congo red, and purpurin 4B proved unsuccessful. When these dyes have been freed from inorganic salts they yield extremely colloidal solutions. No means was found whereby their viscous saturated aqueous solutions could be free from the excess of dye present.

Dye Solubilities at 26° C.

	Water	95% Alcohol
Picric Acid, C. I. No. 7	1.18	8.96
Naphthol Yellow S, C. I. No. 10	8.96	0.025
Aurantia, C. I. No. 12	nil	0.33
Wool Yellow G, C. I. No. 16	18.40	0.24
Chrysoidine Y, C. I. No. 20	0.86	2.21
Chrysoidine R, C. I. No. 21	0.23	0.99
Orange G, C. I. No. 27	10.86	0.22
Chromotrope 2 R, C. I. No. 29	19.30	0.17
Alizarole Yellow GW, C. I. No. 36	25.84	0.04
Alizarole Orange G. C. I. No. 40	0.40	0.57
Azo Bordeaux, C. I. No. 88	3.83	0.19
Metanil Yellow, C. I. No. 138	5.36	1.45
Methyl Orange, C. I. No. 142	0.52	0.08
Helianthin (Color acid of methyl orange)	0.015	0.015
Orange I, C. I. No. 150	5.17	0.64
Orange II, C. I. No. 151	11.37	0.15
Narceine, C. I. No. 152	10.02	0.06
Fast Red A, C. I. No. 176	1.67	0.42
Amaranth, C. I. No. 184	7.20	0.01
Ponceau 6 R, C. I. No. 186	12.98	0.01
Biebrich Scarlet, C. I. No. 280	—	0.05

[1]W. C. Holmes, Stain Solubilities, Part II, Stain Techn. 3, 68, 1927.

DYE SOLUBILITIES AT 26° C.

	Water	95% Alcohol
Bismark Brown Y, C. I. No. 331	1.36	1.08
Bismark Brown R, C. I. No. 332	1.10	0.98
Congo Red, C. I. No. 370	—	0.19
Purpurin 4 B, C. I. No. 448	—	0.13
Sky Blue, C. I. No. 520	13.51	nil
Auramin O, C. I. No. 655	0.74	4.49
Victoria Green (oxalate), C. I. No. 657	7.60	7.52
Pararosanilin (chloride), C. I. No. 676	0.26	5.93
Rosanilin (chloride)	0.39	8.16
Anilin blue (spirit sol.), C. I. No. 689	nil	1.10
Pyronin B (iodine), C. I. No. 741	0.07	1.08
Rhodamine B, C. I. No. 749	0.78	1.47
Ethyl Eosin, C. I. No. 770	0.03	1.13
Thioflavine T, C. I. No. 815	2.11	3.17
Neutral Red (iodide), C. I. No. 825	0.15	0.16
Safranin, C. I. No. 841	5.45	3.41
Methylene Violet RRA, C. I. No. 842	0.69	3.18
Cresyl Violet (NAC)	0.38	0.25
Thionin, C. I. No. 920	0.25	0.25
Methylene Blue ($ZnCl_2$ double salt), C. I. No. 922	2.75	0.05
Toluidine Blue O, C. I. No. 925	3.82	0.57
Alizarin, C. I. No. 1027	nil	0.125
Alizarin, W. C. I. No. 1034	7.69	0.15
Indigo Carmin, C. I. No. 1180	1.68	0.01

A DISCUSSION OF SOME OF THE FACTORS CAUSING VARIABLE RESULTS WITH FLAGELLA STAINS[1]

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ABSTRACT.—**Most flagella stains would probably give more consistent results if they were of stable composition and more was known of the factors influencing the mechanism of their action. Many of the mordants that have been recommended change rapidly, both chemically and physically, after preparation.**

The reaction of some mordants, such as that of Loeffler, seems to be an important factor in their use. It is complicated, however, by temperature effects, oxidizing and reducing agents, the age of the bacterial cultures and variations in different species.

Users of flagella stains will probably always have to select or alter methods to suit the cultures to be stained. Any method which makes use of a minimum of complicated solutions and operations is most likely to give consistently good results.

No clearing or decolorizing agent tried changed poor preparations to good ones, but sometimes good ones can be improved by this treatment.

Altho much has been published concerning the use of dyes in the study of bacteria, no method of staining flagella is available which will give consistent results in the hands of all workers. Since the time of Ehrenberg (1838)[3] many formulae for flagella stains have been recommended by different investigators. The unsatisfactory results that have been secured with all of them, at times, is probably due not to the lack of care taken by workers, but rather to lack of knowledge of the basic factors involved. When we contemplate the difficulties of modern physical chemists in the study of simple aqueous solutions of acids, bases and salts, the futility of explaining the reactions in a heterogeneous mixture of mordants and protoplasm is at once apparent.

Loeffler (1888) was one of the first to stain and photograph the flagella of several species of bacteria. Later (1890) he made the important discovery that the addition of acid or alkali to the mordant

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[2]Most of the detailed laboratory work reported in the experimental part of this paper was done by Miss Ernestine M. J. Long.

[3]For literature cited see bibliography at end of paper.

greatly improved the staining effect with different groups of bacteria. Indeed, when he found the effect of the change of reaction he wrote with characteristic decision:

"Die Lösung des Räthsels schien mir damit gefunden. Die verschiedenen Bakterien verhielten sich derselben Beize gegenüber durchaus verscheiden. Der Alkalizusatz, d.h. das plus oder minus an Säure in der Beize, war das ausschlaggebende Moment. Es handelte sich mithin um äusserst geringe quantitative Differenzen bedingt durch die Natur der betreffenden Bacillen."

Several investigators since Loeffler's time have varied the reaction of the mordants in flagella stains but very few have given it the same careful consideration. Nicholle and Morax (1893) repeated Loeffler's work and found the reaction important but came to the conclusion that control of this factor was too difficult.

Factors of Importance in Methods Which Have Been Used

Reaction

After Loeffler (1890) produced his method, progress was slow because very few realized the importance of knowledge of the flagella of bacteria, and many other factors concerning mordanting and staining were not known.

Loeffler's method was greatly modified by Bunge (1894), and by Loewit (1896). Most of the modifications have been variations in the metal salt and changes in the rate of precipitation of the mordant. More recently heating has been given up and steaming of the mordant has been abandoned. In at least six of the methods listed in Table 1 the effect of reaction can be recognized. See Loeffler (1890), Trenkmann (1896), Moore (1893), Van Ermengen (1894), Williams (see Mallory and Wright, 1924, p. 279), and Shunk (1921). Williams found that an excess of NH_4OH was undesirable while Duckwall (1905), somewhat later recommended the addition of alkali to the mordant. Trenkmann treated the preparations with 1% HCl and tannin solution for six to twelve hours. The slides were then washed in iodine water and stained in gentian violet in anilin water. Scalvo (1893) warmed the mordant and treated the smears with phosphotungstic acid. Zettnow (1899) emphasized the precipitation process and fixed the preparations with formalin adding 0.4% antimony tartrate. Heat was used in this method. Pitfield (see Kendall 1902), Williams, and Casares-Gil (see Galli-Valerio 1915), made use of precipitation with heavy metal salts, tannin and alcohol.

TABLE 1—SUMMARY OF METHODS OF FLAGELLA STAINING

Time of publication	Name of method	Modifications of the method	Oxidation	Reaction	Temperature	Reduction	Precipitation by heavy metal salts
1889	Loeffler	8		Drops of 1% NaOH Drops of 1% H_2SO_4 Basic fuchsin	Steam mordant on slides		Sat. sol. $FeSO_4$
1890	Trenkmann			Bacterial films put in water for 10–12 hrs. 0.5–0.25 cc. 1% HCl used.			Wash with iodine so-solution
1892	Strauss			Ziehl's acid carbol fuchsin			Stain in living condition
1893	Scalvo				Warm the stain		Phospho-tungstic acid
1893	Moore			Basic fuchsin 0.5 cc. of 1% NaOH is added to the mordant	Fix with heat and steam		$FeSO_4$ sat. solution
1893	VanErmengen	4	Osmic acid $AgNO_3$	Glacial acetic acid is added to the mordant	Slides mordanted at 50°C.	$C_2H_3O_2$ & Gallic acid	
1894	Bunge		Expose mordant to the air		Heat sometimes		$FeCl_3$
1896	Loewit			Basic fuchsin, acid alcohol destainer			$CuSO_4$
1898	Bowhill	1	Orcein		Use hot distilled H_2O in mordant. Fix with heat		

TABLE 1—*Continued.*

1899	Zettnow				Fix with heat. Tannin & H_2O on water bath at 35°–40°C. Mordant at 70°–80°C.		Fix with formalin. 0.4% Sb-tartrate is added to get a ppt. Ag or Au afterwards
1899	Morton (MacCrorrie)	1	Night blue		No heat. Warm gently	Adds gallic acid to improve	K alum
1901	Williams		Osmic acid 1% $AgNO_3$ and NH_4OH Ortol photographic developer	Excess NH_4OH interferes. Glacial acetic acid. Wash 0.6% NaCl & 30% NH_4OH			Aluminol $AuCl_3$ & $HgCl_2$
1902	Pitfield	2	Gentian violet		Steam		Sat. sol. alum $HgCl_2$ tannic acid
1915	Casares-Gil (Galli-Valerio)	2	Rosanilin hydrochloride				$AlCl_3$ $ZnCl_2$ tannin, alcohol, 60%
1921	Shunk		Chromic acid alcoholic methylene blue	Acetic acid & KOH		Formalin	Tannin $FeCl_3$ formalin
1926	Gray				No heat		Potash-alum $HgCl_2$

OXIDATION

Many of the solutions that have been used for flagella stains contain oxidizing agents. In the Van Ermengen method osmic acid was used. Gallic acid which is usually considered as a reducing agent was also used. Bunge (1894) exposed the mordant to the air. Bowhill (1898) used orcein and heat. Williams used osmic acid with NH_4OH. The solutions used in the Shunk process contained both osmic acid and formalin. The characteristic brown or black flagella obtained by this method often offer striking pictures.

The tri-phenyl methane dyes used in some methods may be capable of acting as oxidizing agents. The night blue used in the MacCrorrie (see Eyre, 1915, p. 103) method may act in this way. The dye was applied with heat. Morton (1899) found heat to be injurious. However, gallic acid, added after the mordant had been washed from the slide, was very beneficial. In some of the methods, oxidation and reduction may both be taking place.

OTHER FACTORS

In many methods that have been used reaction seems to play an important part, as originally emphasized by Loeffler (1890). It probably has not been given as much consideration as it deserves. Oxidizing and reducing agents have been about as common in many of the methods as substances which influence the reaction of the staining solutions.

The relation of the reaction of solutions containing proteins to the combining power of the proteins with various substances has been pointed out by Loeb (1922). More recently the Stearns (1924) have shown the relation of oxidizing agents to some of the staining processes used with bacteria. The work of Cannan, Cohen and Clark (1926), indicates that the hydrogen ion concentration of solutions is intimately connected with their oxidation and reduction phenomena. These two influences undoubtedly play an important part in the rate and extent of the precipitation of flagella mordants.

Heating is emphasized in the use of many of the solutions that have been used. The rate and extent of the precipitation of most mordants is greatly affected by heating. The uncertain methods that have been used in applying heat often result in over-heating. Such treatment not only results in an unsatisfactory precipitate, but the delicate flagella are often injured or totally destroyed.

The use of young cultures for flagella stains is practically always emphasized. It is well known that very young cultures contain a higher proportion of actively motile cells than older ones. Certainly, the age of the culture is important, because with many bacteria

the percentage of slides showing well stained flagella varies with the age of the culture used.

It may be that the composition of the culture medium used for growing the cultures to be stained affects the results. The production of acid or alkali in the medium might influence the reaction of the cell suspensions to be stained.

Most of the flagella stains that have come into general use employ mordants. Whether mordants are necessary, if all factors could be understood and controlled, is a debated question. Strauss (1892) has reported the staining of flagella in the living condition by means of acid fuchsin. Suspensions of bacteria in the dye were examined in hanging drops. The flagella on the cholera organism and organisms which the author calls *Vibrio avicide* and *Bacillus Finkler-Prior* were seen by this means. More recently Neumann (1925) has seen and photographed the flagella of living bacteria without any staining treatment whatever. The bacteria are held motionless in a 7% gelatin solution, and viewed in a dark-field, illuminated by a dark-field condenser of special design.

Careful examination of good flagella stains, made by any of the methods where mordants are used, shows the field surrounding the bacteria to be covered with a very fine grained film. The film may be entirely clear or quite deeply stained, depending upon the method that has been used. This film may be seen to be present before the regular stain is applied. In the film the flagella may be seen by their bluish or black appearance. It has sometimes been assumed that the mordant becomes precipitated on the flagella, thus rendering them visible, and causing the stain to adhere to them. It seems just as possible that there is also a chemical combination of the mordant with the flagella which actually stains them, increasing their visibility. The after treatment with a dye only increases this effect.

Many of the mordants are prepared in alcoholic solutions. Most of them are probably more soluble in alcohol, but the alcohol and dye often used may cause a swelling of the flagella and form a compound with them. When 95% alcohol is used in the Casares-Gil mordant, the flagella do not seem to be affected but the cells are swollen and distended. When 60% alcohol is used, swelling does not take place.

A number of the procedures for staining flagella include the final treatment of the preparations with decolorizing and clearing agents. The use of alcohol, xylol, and oils for this purpose often improves the appearance of the preparations by removing or rendering invisible troublesome precipitates. On the other hand decolorizing

agents, unless very cautiously used, tend to destain the flagella and reduce the brilliancy of the preparations.

EXPERIMENTAL WORK

The limited amount of data presented here is gleaned from hundreds of preparations stained by three methods. Modifications of the original Loeffler method were compared with the method used by Gray (1926). The modification of the Casares-Gil method recommended by Thatcher (1926) was used for most of the experiments.

PREPARATION OF CULTURES

The microörganisms used for the experiments were *Bacillus tritus* Batchelor, *Bacillus amylovorous* (Burrill) Trev. (the fire-blight organism) and *Bacillus radiobacter* Beij.[4] The only reasons for using these microörganisms were that they were available and showed very active motility under average laboratory conditions.

Several successive transfers were always made in liquid media and the last one streaked on hard agar slants. The cultures were placed at 30°C. and watched for growth. At the earliest signs of well developed growth, 20 to 24 hours, suspensions were prepared in sterile distilled water.

PREPARATION OF SMEARS

The author agrees with everything that has previously been written about using clean cover glasses or slides. All of the experiments reported here were made with smears upon slides.

New slides were first cleaned in sulfuric-acid-dichromate cleaning mixture and finally washed in distilled water followed by 95% ethyl alcohol made alkaline with 1% ammonium hydroxide.

Smears were prepared from suspensions of the organisms which had stood for a short time at room temperature. Gray's method of making smears was used at all times. Smears were spread so as to cover about three-fourths of the area of the slides.

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[4]The culture of *Bacillus tritans* was isolated in the writer's laboratory. It was checked carefully with the published descriptions of this and similar organisms, and agreed with the description of this species given in the last edition of Bergey's Manual of Determinative Bacteriology (Williams and Wilkins, Balto. 1925). The culture of *B. amylovorous* was obtained from the Dept. of Plant Pathology of the University of Wisconsin and gave the characteristic reactions for this organism. The culture of *B. radiobacter* was secured from the soil bacteriological laboratory of the Bureau of Plant Industry of the U. S. Dept. of Agriculture; its reactions agreed with published descriptions.

PREPARATION OF MORDANTS

The effect of several salts of heavy metals on the kind of precipitate was studied. Loeffler's mordant was used. It was hoped that salts of other metals substituted for ferrous sulfate might give better results. Cobalt, nickel, mercury, zinc, manganese, magnesium and cupric chlorides were tried in saturated solutions. Chromium nitrate and a 45% solution of cobalt chloride were used. The results are given in Table 2.

When these solutions were used for flagella staining they all gave very poor results. Smears made from the same suspensions at the same time stained with a ferrous sulfate mordant gave good results. The mordant of the Casares-Gil method was used as recommended by Thatcher. The Gray mordant was used as directed by the author.

TABLE 2. EFFECT OF HEAVY METAL SALTS ON TANNIN AND ZIEHL'S CARBOL FUCHSIN IN THE LOEFFLER MORDANT

Metal Salt	Action on Tannin	Action on Tannin plus Fuchsin
$FeSO_4$ sat. sol.	Heavy black ppt.	Very fine ppt. in susp.
$CoCl_2$ sat. sol.	Coarse pink ppt.	Ordinary ppt.
$CoCl_2$ 45% sol.	Coarse pink ppt.	Ordinary ppt.
$NiCl_2$ sat. sol.	Clear green sol.	Ordinary ppt.
$HgCl_2$ sat. sol.	Slight ppt.	Finely divided ppt.
$ZnCl_2$ sat. sol.	Heavy white ppt.	Very light red ppt.
$MnCl_2$ sat. sol.	No ppt.	Ordinary ppt.
$MgCl_2$ sat. sol.	Fine, white ppt.	Light red ppt.
$Cr(NO_3)_3$ sat. sol.	Dark, clear fluid	No ppt.
$CuCl_2$ sat. sol.	Dark, green, clear	Slight ppt.

TEMPERATURE EFFECTS

Several series of slides were mordanted at different times under different temperature conditions. None of the smears were ever fixed by heat. In all cases the temperature of the mordant was taken and not the temperature of the bath. Preparations were mordanted at 25°C., 30°C., 35°C., and 37°C. Temperatures above 30°C. increased the rate of precipitation and caused great clots to form on the slides, even when in a vertical position. The best results were obtained at 25°C. with the Casares-Gil mordant. Loeffler's mordant gave good results at higher temperatures.

COUNTERSTAINS

Many workers have found that it is not entirely necessary to use carbol fuchsin as a counterstain. Other counterstains seem to give equally good results. These experiments gave the best results when

Ziehl's carbol fuchsin was used. Methylene blue and crystal violet were used with some success. Crystal violet affects the rate of precipitation and the slides are generally not satisfactory. Methylene blue stains too faintly.

EFFECT OF REACTION ON THE MORDANT

Control of the reaction of staining solutions is a difficult matter. The use of the usual colorimetric methods is impossible. Potentiometric measurements have to be made. Very great changes in the reaction of most mordants cause excessively heavy precipitates to form. The effect is to "poison" the electrodes and render the determinations inaccurate or of doubtful value.

Variations of the reaction of the Loeffler and Casares-Gil mordants were tried. All pH-values were determined with the hydrogen electrode and a Leeds and Northrup potentiometer. Readings were made when the mordants were used and each electrode was used only once. The mordants were used at a temperature of 25°C. Other conditions were the same as in previous experiments.

A few experiments soon showed that it is not possible to shift the reaction of the Casares-Gil mordant very far and have it retain its staining properties. The normal reaction of all batches of this mordant was close to pH 2.08. A few drops of dilute sodium hydroxide always produced a heavy precipitate which would not stain flagella.

The narrowness of the pH-range of this mordant seems to support the contention that it is a balanced colloid as prepared. This may be one reason why it has given good results repeatedly in the hands of different workers. On the other hand, if its reaction can not be changed without spoiling it, there may be some bacteria, the flagella of which cannot be stained with it.

The Loeffler mordant can be varied over a greater pH-range. The initial pH of this mordant was usually near 2.3. It changes on standing and may drop below 2. Near pH 7 it becomes a semi-clear liquid and has no staining properties.

Oxidizing agents were used on the smears with both methods but very little effect could be noticed.

The reaction of the Loeffler mordant was varied over a range from pH 1.56 to 5.48. The details of the staining process were carried out as before. The results with *B. radiobacter*, *B. amylovorous* and *B. tritus* are given in Tables 3, 4 and 5 respectively. In no case with sets of approximately 100 each did all slides show flagella. The results of the grading of the quality of the preparations are based only upon slides showing some flagella. In order to place

the results on a percentage basis, the poorest slides, showing only a few faintly stained flagella, were classed as none. Any method of measurement based upon individual judgment is never satisfactory, but in this case was the only means available.

TABLE 3. EFFECT OF REACTION ON THE STAINING POWER OF LOEFFLER'S MORDANT WITH *B. radiobacter*.

pH	Quality of Preparations			
	Excellent	Good	Poor	None
	%	%	%	%
1.56	0	0	8	92
1.62	0	0	16	84
1.77	0	8	8	84
1.95	0	0	8	92
2.06	0	0	0	100
2.30	0	0	0	100
2.38	0	0	16	84
4.79	0	0	8	92
5.48	0	0	8	92

TABLE 4. EFFECT OF REACTION ON THE STAINING POWER OF LOEFFLER'S MORDANT WITH *B. amylovorous*.

pH	Quality of Preparations			
	Excellent	Good	Poor	None
	%	%	%	%
1.56	0	0	9.9	90.1
1.62	0	9.9	19.8	70.3
1.77	0	9.9	0	90.1
1.95	0	9.9	0	0
2.06	0	19.8	9.9	70.3
2.30	0	0	0	100
2.38	0	0	0	100
4.79	0	0	9.9	90.1
5.48	0	0	9.9	90.1

The best slides of all three series were taken as standards for comparison. The greatest number of such slides were preparations of *B. tritus* mordanted at pH 1.77. The depth of color in the flagella was taken as the standard of excellence rather than freedom of the field from precipitate.

The results given in Table 3 show that no good flagella preparations of *B. radiobacter* were secured when the reaction of the mordant was at pH 2.06 and 2.30. Tables 4 and 5 show no good

preparations of *B. amylovorous* and *B. tritus* obtained when the mordant had a reaction of 2.30 and 2.38. In every case the most satisfactory preparations were secured at more acid reactions.

Such marked failure to stain the flagella of certain bacteria at definite pH-values is in accord with the more recent views of Loeb (1922) in regard to the chemical behavior of proteins. If such is the case, mordants must not only precipitate on the flagella of bacteria, but they combine with the flagella only when the reaction is off the isoelectric range. Judging by these limited experiments, the most favorable reaction seems to be on the acid side of the isoelectric range with the organisms used.

TABLE 5. EFFECT OF REACTION ON THE STAINING POWER OF LOEFFLER'S MORDANT WITH *B. tritus*.

pH	Quality of Preparation			
	Excellent	Good	Poor	None
	%	%	%	%
1.56	0	0	17	83
1.62	0	17	0	83
1.77	34	17	0	49
1.95	0	0	17	83
2.01	0	17	0	83
2.06	34	0	17	49
2.30	0	0	0	100
2.38	0	0	0	100
2.86	0	17	0	83
4.21	0	0	17	83
4.79	0	0	17	83
5.48	0	0	17	83

CLEARING AND DECOLORIZING AGENTS

The Casares-Gil method gave the most consistent results but the preparations nearly always showed considerable heavy precipitate. This can be decreased by dilution of the mordant which also decreases its staining power. Precipitate is of no disadvantage in flagella preparations for direct examination as in taxonomic and diagnostic work. However, when demonstration mounts or photomicrographs are wanted, a clear field is of primary importance. Fields of remarkable clearness and brilliance have been secured at times with most methods. The procedures of Loeffler and Gray nearly always give clear fields even when the flagella are well stained. The after treatment of the preparation with alcohol and xylol as recommended in some methods probably helps to remove or render

invisible unsightly precipitate. Such treatment may a'so reduce the brilliancy of the stained flagella.

Some experiments were made with various decolorizing and clearing agents. Specimens showing well stained flagella by the Casares-Gil method were treated with ethyl alcohol of various concentrations, xylol, benzine, anilin oil, clove oil and origanum oil. All except origanum oil were of very little value. Most decolorizing agents either ruin the preparations or decolorize the flagella as well as the precipitate.

After frequent trials a mixture of equal parts of oil of origanum and absolute alcohol was found to improve preparations. Some practice is necessary before good results can be obtained. Preparations with excessive precipitate can never be cleared until the background is entirely clear. It is best to destain only for a few seconds, transfer to xylol, dry and examine. The operation can be repeated if desired. Photomicrographs showing the effect of reaction and decolorizing agents may be seen in the accompanying plate.

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EXPLANATION OF THE PLATE

All of the photomicrographs were made with Leitz 1/12a oil immersion objective 15X "Periplan" ocular and immersed condenser N. A. 1.40 at a tube length of 170 m.m. The Leitz Mikro-Aufsatzkamera, "Makam" was used. Magnifications 1500X.

FIG. 1. *B. tritans*, Loeffler flagella stain pH 2.06.

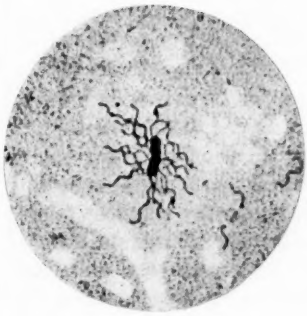
FIG. 2. *B. tritans*, Loeffler flagella stain pH 2.30.

FIG. 3. *B. tritans*, Loeffler flagella stain pH 2.86.

FIG. 4. *B. tritans*, Casares-Gil flagella stain not destained.

FIG. 5. *B. tritans*, Casares-Gil flagella stain destained with origanum oil.

FIG. 6. *B. tritans*, Casares-Gil flagella stain destained with a mixture of one part absolute alcohol and one part origanum oil.



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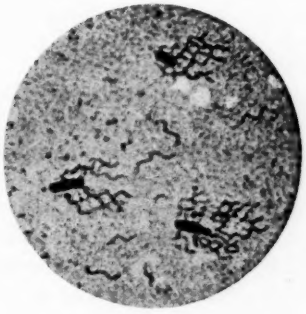

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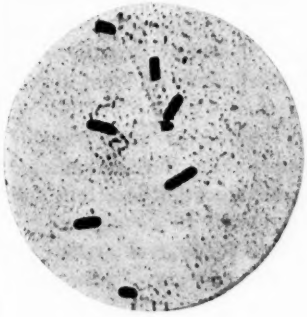

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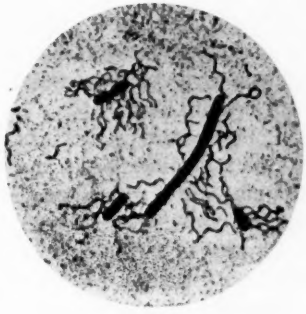

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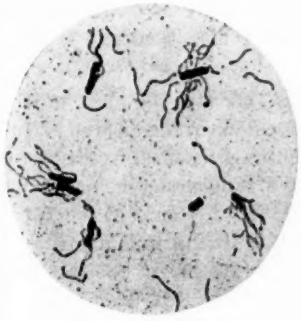

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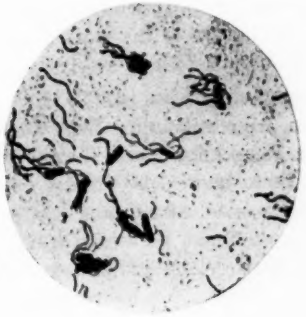

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METALLIC LAKES OF THE OXAZINES (GALLAMIN BLUE, GALLOCYANIN AND COELESTIN BLUE) AS NUCLEAR STAIN SUBSTITUTES FOR HEMATOXYLIN

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Abstract:—**Becher's investigations upon the soluble metallic lakes of the oxazines have been re-investigated, extended and results described. Gallamin blue, gallocyanin and coelestin blue in combination with ferric ammonium sulfate gave the best results. The dyes are dissolved in a five per cent aqueous solution of ferric ammonium sulfate. The solution is boiled for 2–3 minutes, cooled, filtered and ready for immediate use. The iron lakes of these dyes stain nuclei excellently giving a deep blue or blue black in 3–5 minutes. No differentiation with acid is required. Coelestin blue gives the most stable solution and is recommended as a routine nuclear stain. The protoplasm remains practically colorless and counter-staining with acid dyes such as ethyl-eosin, orange G, or fuchsin gives pictures which cannot be distinguished from a good hematoxylin stain.**

Counter-staining with van Gieson solution is also possible. Benda's modification of the van Gieson solution is recommended. Staining of fat with Sudan, scarlet red, etc., does not interfere with nuclear staining by these dyes.

As applied to the central nervous system these dyes are far superior to hematoxylin. Ganglion and glia cells are as excellently stained as with thionin.

The most widely used fixatives, namely formaldehyde, Mueller-formaldehyde, Zenker's and alcohol, give equally as good results. The nature of the staining process is briefly discussed and a prospectus offered.

Hematoxylin, and to less extent carmin, are still the most widely used nuclear stains. Both are natural dyes, the exact chemical constitution not known, and they have not been entirely replaced by synthetic anilin dyes. The difficulty in obtaining a satisfactory hematoxylin as well as carmin is generally recognized. H. J. Conn[1] has recently pointed out in this journal the difficulties in the manufacture of hematoxylin to obtain a product sufficiently uniform to meet the requirements of histologists. The anilin dyes, methyl green, methyl violet, thionin, methylene azure, are excellent nuclear dyes, but they are not fast colors. Safranin has somewhat greater resistance

[1]H. J. Conn. The Hematoxylin Problem. Stain Techn. 2, 1–3. 1927.

but has certain drawbacks in general use. It is useful only for specimens fixed with chromic-osmic acid mixtures. The above mentioned anilin dyes are easily extracted with dilute alcohol and acid dyes, especially where acid counterstains for cytoplasm are used. Furthermore, these anilin dyes are easily reduced to their leucobases by the action of balsam and usually fade in a few months.

Hematoxylin stands in contrast and is strongly anchored to the nuclear substances and practically unchanged by the acid cytoplasmic counter-stains. Hematoxylin and carmin also display a much greater resistance to reducing and oxidizing processes. Sections stained with carmin may keep for ten years or longer, if not indefinitely. The same applies to iron hematoxylin. Alum-hematoxylin is more susceptible. Its solution is not very stable and stained specimens may show a brown discoloration. Possibly the balsam acts as an oxygen carrier, and the increasing acidity of the balsam after drying may also have a part in the discoloration.

Carmin is by no means an ideal stain. The intensity and distinctness with which it brings out the nuclei depends a great deal upon the mode of fixation. The red is less distinct than a blue or bluish-black color. It is practically abandoned today for finer cytological work. It is especially useful for staining tissue en block for topographical cell studies.

From this it is obvious that new nuclear dyes to compete with hematoxylin and carmin are desirable. Very little attention has been paid by histologists to the excellent work of Becher[2] on the use of certain groups of anilin dyes in combination with metal salts. These give nuclear stains which, in brilliancy and resistance, are superior to hematoxylin and carmin. Becher found that the oxyanthraquinon dyes are the most suitable. They give soluble lakes with certain metal salts.

The anthraquinon and naphthoquinon dyes are mordant dyes and are used extensively in the textile industry. They are fast dyes, insoluble in water, alcohol, acid, and alkalies, and very resistant against oxidation and reduction. There are exceptions to the difficulty with which oxidation can take place in the cases of anthragallol and rufigallol. These contain the gallic acid complex (similar to the pyrogallol) and oxidize more easily. Resistance to oxidation protects the dye solution from over-ripening and at the same time from discoloration of stained sections. The reduction

[2]Becher, Siegfried. Untersuchungen über die Echtfärbung der Zellkerne mit künstlichen Beizen-farbstoffen und die Theorie des histologischen Färb-prozesses mit gelösten Laken. Berlin, Verlag von Gebrüder Borntträger. 1921.

fastness of the oxyanthraquinon dyes can be easily demonstrated by heating the dyes in 5% solution of hydro-sulfite. This reagent will bleach most dyes while anthragallol, alizarinocyanin, and anthracene blue are not affected at all. Alizarin red, alizarin orange, alizarin granat, alizarin bordeaux are given a faint brown tinge by the treatment.

Oxyanthraquinons contain two to eight hydroxyl groups. The most valuable technical dyes have at least two of these in the ortho-position in the neighborhood of the ketone group. These stains have a weakly acid character like carminic acid or hematoxylin, and a greater affinity for cytoplasm than nuclei. Alizarin dissolved in 50% acetone stains cytoplasm and nuclei diffusely and brings the latter out fairly well. A basic mordant forming a so-called *lake* must be added to bring the structures out distinctly and clearly. In this way the nucleus is made most prominent while the cytoplasm appears unstained or very faintly colored. As mordants for this purpose basic metallic oxides are used. An example is aluminium oxide which is used exclusively in the textile industry. Here alum is combined with a basic (soda) or a slightly hydrolyzing substance (such as a tartaric acid) after which aluminium oxide is precipitated upon the fiber. Dyeing of fibers is a dichronous process, the substance being immersed separately in mordant and dye. This gives a diffuse stain of the fiber.

The histologist requires a differential stain and cannot directly copy the technical textile process. The mechanism of staining by hematoxylin for histological purposes is a different one. The acid hematoxylin is dissolved in a basic mordant (alum) forming a soluble lake with basic properties which is anchored by the nucleic acid of the tissue, resulting in the differential staining of the nuclei. In other words, the basic hematoxylin with alum is anchored to the nuclei as such. The metal compound of the dye, the lake, must be soluble. Mordant staining in histology is principally staining with soluble lakes.

The usual hematoxylin and carmin stains are such soluble lakes. A dichronous staining process is the iron hematoxylin staining method of Heidenhain. Sections are first mordanted with iron alum and then immersed in the pure hematoxylin solution. It may be that the mordant is localized and the hematoxylin, secondarily anchored, produces a diffuse stain. By this differentiation of the section with iron alum we make use of the solubility of the lake in the iron alum (mordant).

Staining with soluble lakes seems contrary to accepted methods because the lakes are in general considered as insoluble compounds.

Becher has shown that there is no essential difference between the anthraquinon lakes, hematoxylin, carmin, and gallein lakes. The most valuable finding in Becher's investigation is that oxyanthraquinons and naphthoquinons form soluble lakes which are excellent nuclear stains and in the writer's opinion, will replace hematoxylin in the future.

Since Becher's publication of his original monograph which contains a wealth of information and new suggestions for the histologist, only C. Krauspe[3] and L. Siegel[4] have investigated his claims as to the usefulness of the soluble lakes as nuclear dyes. Krauspe used gallocyanin in a 5% aqueous solution of chrome alum and obtained a distinct staining of nuclei in 12 to 14 hours. Siegel used coelestin blue, gallamin blue, gallocyanin, and alizarin cyanin with the solvent of 50% alcohol or 5% aqueous solution of sodium alum or chrome alum. Gallocyanin with chrome alum or alizarin cyanin and sodium alum stained the nuclei distinctly in 20 to 24 hours. Coelestin blue seems most useful and stained the nuclei in about one hour. Siegel comes to the conclusion that Becher's soluble lakes are not suitable for human tissue.

The excellent results obtained in our first attempts with gallamin blue, gallocyanin and coelestin blue furnished the impetus to reinvestigate the claims of Becher[5] in spite of the discouraging results of Siegel. Becher used sections of *Salamandra maculosa* fixed in bichloride alcohol. Borax and boric acid separately and in combination, aluminium potassium sulfate, sodium potassium sulfate, chromium potassium sulfate, ferric ammonium sulfate and ferric sulfate were used as mordants. The results of Becher's staining experiments with these dyes may be summarized as follows:

Aqueous solutions—The dyes stain only the ground substances of cartilage (gallocyanin and coelestin blue) or the muscle fibers (gallamin blue). The latter is of practical importance.

Borax lakes—The solutions give nuclear staining but these are too weak for practical purposes. Cytoplasmic structures are also stained with this mordant. Gallamin blue is especially valuable for tissues with high calcium content as acid solutions dissolve the calcium.

[3]Krauspe, Karl. Gallocyanin (Becher) als Kernfarbstoff, nebst einigen Bemerkungen über das Färben und Versilbern von Gelatinschnitten. Centbl. f. allg. Path. und path. Anatomie. 32, 392.

[4]Siegel, L. Über die verwendbarkeit künsticher Beizenfarbstoffe in der menschlichen Histologie. Centbl. f. allg. Path. und path. Anatomie. 34, 228. 1924.

[5]These methods are available not only to dyes named herein but to many others as well. Thiazins, tri-phenyl methanes, azo dyes, oxyketones, etc., have been successfully used.

Potassium or sodium alum lakes—Gallamin blue gives the best results. Cartilage and nuclei are distinctly and metachromatically stained. Gallocyanin and coelestin blue stain nuclei less strongly, the latter giving a strong metachromatic color to connective tissue.

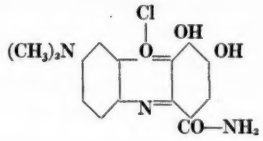
Chrome alum lakes—Coelestin blue gives results similar to those with potassium alum. Gallamin blue stains nuclei and muscle tissue distinctly. Gallocyanin gives excellent results in staining nuclei a pure deep blue.

Iron lakes—(ferric ammonium sulfate and ferric sulfate)—Gallocyanin stains nuclei distinctly and is very useful. Gallamin blue and coelestin blue give good nuclear stains.

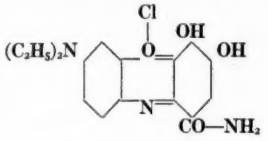
The purpose of our investigations, the details of which are represented in the condensed tables following, was to find suitable metal salts with which these dyes would give useful lakes to the end that hematoxylin could be replaced to advantage in the routine staining of human tissue. It was desired, moreover, that this should not interfere with counter-staining with acid plasma dyes. Becher's investigations indicate that speed and selection are best obtained with iron and chromium lakes. Aluminium lakes, while giving excellent results, stain too slowly. We have extended Becher's observations as noted in using aluminium, arsenic, boron, calcium, chromium, cobalt, copper, gold, lithium, manganese, mercury, molybdenum, platinum, silver, strontium, tungsten, uranium, vanadium, and zinc in our experiments.

The dyes used in our staining experiments were gallamin blue (Bayer) gallocyanin (Bayer), and coelestin blue B (Bayer) and can be obtained from Grübler, Leipzig. The chemical formulae and their chemical and physical characteristics are given below.

Gallamin blue

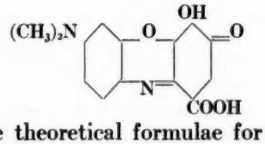


Coelestin blue B

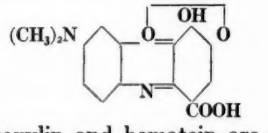


Gallocyanin

o-quinoid

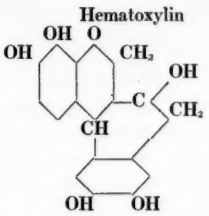


p-quinoid

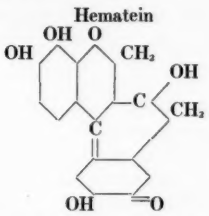


The theoretical formulae for hematoxylin and hematein are given here for contrast:

Hematoxylin



Hematein



Gallamin blue is obtained commercially as a gray paste or as dry amorphous powder. It is soluble in warm water to which it gives a deep blue color. In hydrochloric acid or sodium hydroxide the solutions are red or red-violet respectively. In concentrated sulfuric acid it is soluble with a red color. Cotton mordanted with chromium and tartaric acid is stained blue by the dye. The spectrum gives a value for λ of 650.75.

Gallocyanin is obtained commercially as the free base in the form of crystals of greenish color or as a chlorhydrate, a greenish powder soluble in warm water with a violet color. It is also fairly soluble in alcohol with a blue-violet color. In hydrochloric acid or sodium hydroxide the dye is soluble with fuchsin red or red violet color respectively. In concentrated sulfuric acid the color is deep blue.

TABLE 1. STAINING RESULTS WITH GALLOCYANIN.

Metal salt (mordant)[1]	Nuclei	Other cellular elements
Chromium potassium sulfate[3]	Distinct blue gray	Colorless
Ferric acetate	" gray blue	"
Ferric ammonium sulfate[2]	" light blue	"
Ferric chloride	" light blue	"
Ferrous nitrate[3]	Pale, gray-blue	"
Ferrous sulfate[3]	Pale, gray	"

[1]The following metal salts were used without success:—aluminium ammonium sulfate, aluminium chloride, aluminium potassium sulfate, calcium chloride, boric acid in combination with sodium borate, sodium borate, cupric acetate, cupric chloride cupric nitrate, ferric ammonium oxalate, ferric nitrate, ferric oxalate, ferrous chloride gold chloride, silver nitrate, sodium tetravanadate, sodium tungstate, platinum chloride, vanadium sulfate.

[2]Zenker fixation gave the same result. When only 1% of the metal salt is used the nuclei are deep blue and excellently differentiated.

[3]Tried also upon kidney tissue.

It is easily reduced to its leuco-base in alkaline solution. Wool or cotton mordanted with chrome are dyed a blue-violet with gallocyanin. The value of λ for the water spectrum is 635.4

Coelestin blue B comes commercially as a greenish-black powder, soluble in water with a blue color. It is soluble in alcohol with a

TABLE 2. STAINING RESULTS WITH GALLAMIN BLUE

Metal salt (mordant)[1]	Nuclei	Other cellular elements
Aluminium chloride[2]	Distinct, blue gray	Slightly blue-gray
Aluminium fluoride[3]	Heart, Not stained	Deep blue
	Brain, Pale blue	Blood vessels distinct dark blue
Ammonium chromate[3]	Heart, Not stained	Light green
	Brain, Not stained	Not stained
Antimony tartrate[3]	Heart, Not stained	Deep blue. conn. tiss.
	Brain, Distinct, blue	Bld. vess. distinct, dark blue
Atoxyl[3]	Not stained	Only bld. vess. stained
Chromium pot. sulfate[4]	Distinct, pale blue	Colorless
Cobaltous chloride[3]	Heart, Pale blue	Deep blue
	Brain, Pale blue	Blood vessels distinct, dark blue
Cupric chloride[3]	Heart, Not stained	Deep blue
	Brain, Distinct, deep blue	Light blue
Cupric nitrate[3]	Heart, Not stained	Not stained
	Brain, Very distinct	Not stained
Ferric alum. sulfate[5]	Deep blue	Diffuse deep blue
Ferric ammon. sulfate[6]	Distinct, blue gray	Not stained
Ferrous sulfate[3, 7]	Deep blue-black.	Conn. tiss. pale gray-blue, excell. musc. stain (dk. blue)
Ferrous nitrate[6]	Distinct, dark blue	Neuroglia gray-blue
Ferrous chloride[6]	Same excellent	Neuroglia pink
Lithium sulfate[3]	Heart, Not stained	Deep blue
	Brain, Pale blue	Blood vessels deep blue
Strontium chloride[3]	Same	Same
Manganese chloride[3]	Heart, Not stained	Light blue
	Brain, Not stained	Not stained
Sodium tetravanadate[3]	Heart, Not stained	Not stained
	Brain, Not stained	Only bld. vess. stained
Sodium tungstate[8]	Distinct, pale blue	Colorless
Phosphomolybdic acid[3]	Heart, Not stained	Pale green-blue
	Brain, Pale green	Bld. vess. distinct green
Phosphotungstic acid[3]	Heart, Faintly red	Carmin red
	Brain, Pale red	Blood ves els blue-violet
Uranium acetate[3]	Heart, Not stained	Pale blue
	Brain, Red violet	Indistinct
Uranium nitrate[3]	Heart, Not stained	Pale blue
	Brain, Distinct	Colorless

[1]Other metal salts used without success were:—aluminium nitrate, aluminium ammon. sulfate, boric acid in combination with sodium borate, calcium chloride, cupric chloride, cupric acetate, cupric sulfate, cadmium sulfate, ferric chloride, ferric phosphate (1%), ferric ammon. citrate (1%), ferric nitrate, ferric oxalate, ferric ammon. oxalate, ferric acetate, ferric nitrate, ferrous sulfate, ferrous nitrate, ferrous

blue color, in hydrochloric acid with a red, in sodium hydroxide with a blue-violet, and in sulfuric acid with a blue color. Cotton mordanted with chromium stains a violet with coelestin blue B. The color is fast to light, acid, and alkalies. The λ value in water solution is 654.45, 600.20, and 552.60.

TABLE 3. STAINING RESULTS WITH COELESTIN BLUE B.

Metal salt (Mordant)[6]	Nuclei	Other cellular elements
Chrom. pot. sulfate[1, 2]	Excell. deep blue	Neuroglia not stained
Ferric ammon. sulfate[1, 3]	Distinct blue-gray	Not stained
Ferric acetate[4]	Deep blue	Pink
Ferric ammon. sulfate	Deep blue	Diffuse
Ferrous sulfate[2]	Excellent differentiation	Pale pink
Ferrous nitrate[5]	Distinct-blue-gray	Pink
Ferrous oxalate[5]	" " "	Red protoplasm and connective tissue
Sodium tungstate[3]	Distinct pale blue	Not stained

[1]Methyl alcohol fixation gave the same result.

[2]Mueller-formol fixation. 1% metal solution. With formaldehyde fixation and 1% metal salt the nuclei are distinct and connective tissue a metachromatic pink. With Zenker or methyl alcohol fixation the nuclei are gray-blue and excellently differentiated. These sections from Zenker or formol could be kept only in glycerin. Brain tissue could not be used.

[3]Zenker fixation.

[4]Kidney tissue used. One hour staining.

[5]Kidney tissue used.

[6]Other metal salts tried without success were:—aluminium chloride, calcium chloride, ferric phosphate, ferric ammon. citrate, ferric ammon. sulfate, ferric nitrate, ferrous ammon. sulfate, ferrous sulfate, ferrous chloride, ferrous ammon. oxalate gold chloride, platinic chloride, silver nitrate, vanadium sulfate.

A number of tests with these dyes and metallic mordants have been made in this laboratory and are described in the tables following. Unless otherwise noted the preparations should be assumed to be from brain tissue fixed in formaldehyde and the metallic salt used

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chloride, gold chloride, mercuric chloride, sodium fluoride, sodium nitro-prusside, sodium tetravanadate, potassium alum, platinic chloride, silver nitrate, zinc acetate. and vanadium sulfate.

[2]Not good except for deep blue nuclei with Zenker and Mueller formol fixation.

[3]Heart and brain used.

[4]Zenker fixation. (Not good with formol fixation).

[5]Zenker fixation. Nuclei excellently stained a deep blue. The rest is a light blue, myelin sheaths unstained.

[6]Mueller-formol fixative. Formol fixation not good.

[7]One percent used.

[8]Zenker fixation.

in 5% solution with 0.5% of the dye.[6] Tissues were stained in general from 3 to 5 minutes. One is often sufficient.

Coelestin blue, gallamin blue, and gallocyanin in combination with ferric ammonium sulfate gave the best results. The iron lakes of all these dyes stain nuclei excellently, giving deep blue or blue-black in 3 to 5 minutes. The protoplasm remains practically colorless and counter-staining with acid dyes such as eosin, orange G, fuchsin, etc. gives pictures that cannot be distinguished from a good hematoxylin stain. Counter-staining with van Gieson solution is also possible by either the original method, or, better, with Benda's modification in which the picric acid is replaced by ammonium picrate[7]. If the original van Gieson solution is used the section should be counterstained for one-half minute only or the nuclei appear greenish-black. With Benda's modification of the van Gieson solution, staining can be prolonged for 10 to 15 minutes when the nuclei appear almost deep black. In using coelestin blue the ground substance of cartilage is stained a metachromatic pink. Gallamin blue and gallocyanin give equally good results. The former stains muscle fiber a deep blue, but neither dye forms a very stable solution. After two weeks they lose their staining power. Where rapid staining is not required they may be used for several weeks. Coelestin blue gives with ferric ammonium sulfate a most stable solution. This can be used for several months. Some of the dye will be precipitated but this does not interfere with the staining. We have kept the staining solution in open jars for several months. If the water evaporates the dye may be diluted with distilled water and boiled for a few minutes.

The great advantage of the iron alum combination with any one of these dyes is in the excellent differentiation of the chromatin. No differentiation with acid is required. The so'ution can be easily made in a few minutes and is ready for immediate use. Coelestin blue is cheaper than hematoxylin and variations in manufacturer's products or different batches do not interfere with the staining. This is not the case with hematoxylin. The staining of fat with sudan, scarlet red, or oil red does not interfere with nuclear staining by these dyes.

As applied to the central nervous system these dyes are by far

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[6]The solution of the metal salt and dye is brought to a boil and allowed to cool, after which it is ready for use.

[7]Use 95 cc. of a saturated aqueous solution of ammonium picrate, 5 cc. 1% aqueous solution of acid fuchsin.

For staining add to 10 cc. of this solution 2 drops of a saturated aqueous solution of picric acid.

superior to hematoxylin. Ganglion and glia cells are as excellently stained as with thionin. The most widely used fixatives, namely, formaldehyde, Mueller-formaldehyde, Zenker's fluid, and alcohol give equally good results.

The dichronous staining of sections by immersion first in a solution of metal salts and then in an aqueous solution of one of these stains has so far given poor results. Re-immersion of overstained sections in the metal salt solution results in a complete decolorization of the section. For this reason coelestin blue, gallamin blue and gallocyanin are so far unable to replace hematoxylin for the differential staining of myelin sheaths.[8] Further experiments with different fixatives and use of other metal salts may give the desired results.

As concerns the nature of the staining reaction or process, Becher has expressed the opinion that a soluble lake is anchored as such to the chromatin in the form of a complex metal salt. He does not believe that the metal salt first combines with the chromatin between which and the dye it then acts as an amboceptor, so to speak. Hematoxylin, on the other hand, seems to be anchored directly to the metal which has already been fixed to the tissue. The latest investigation of Gustein[9] indicates that the hematoxylin stains the lipoids, or better, the lipo-proteins, and it is not directly combined with the chromatin. After extraction of sections with acid alcohol, staining with hematoxylin is not possible. If these investigations are correct, there is a difference in the staining properties of the soluble lakes of the oxazines and hematoxylin stains. The two processes may be the same, however, should the metal act in either case as an amboceptor between the lipoid and the dye. Hematoxylin is apparently not a specific stain for chromatin but a lipoid dye. The oxazines appear to stain chromatin directly. Further investigation will clarify this point. In this connection it is of interest to compare these results with those recently obtained by us[10] in the staining of iron in the tissues with anilin dyes. This staining is probably based upon the same principles as that described above, the iron or other metal within the tissue providing the necessary metal salt for the lake formation.

In conclusion we wish to call attention to the flexibility of adaptation permitted by these staining methods which is not afforded by

[8]Acid black may be able to perform this staining (see Virchow's Archiv. v. 261, 1926 (853).

[9]Virchow's Archiv. v. 261, 1926 (846).

[10]American Journal of the Medical Sciences. Not yet published.

hematoxylin. Many metal salts reported as valueless from the results of our experiments will doubtless be found of considerable value in other cases. It should also be noted that staining periods exceeding ten minutes have seldom been used in this investigation. Longer periods should bring out more valuable combinations.

NOTES ON TECHNIC

METHOD OF PREPARING SLIDES FOR CLASS USE. Those who have classes using prepared slides know how difficult it is to keep slides of sectioned material in good condition. This is especially noticeable where several laboratory sections use the same slides. A complete set of good slides may be put into the laboratory and in a short time with rough usage some of the slides will be useless on account of crushed sections. The toll is especially heavy in embryology classes using chick slides where the delicate extra-embryonic membranes and other structures are so easily broken by slight pressure on the cover-glass. In view of these difficulties it is apparent that any increase in the durability of slides is quite worth while. The writer has done some experimenting with the celloiden coating of chick sections, and a method has been developed which has proved very successful. Celloiden has long been used in slide making, but this particular method does not seem to have been reported before. The slides are prepared in the following manner:

1. Prepare the sections and dehydrate to absolute alcohol in the usual way. Remove the slide from the alcohol and while it is held in a horizontal position apply a few drops of thin celloiden with a pipette, allowing it to flow evenly over the sections.

2. To harden the celloiden lay the slide on pieces of broken glass or other support in a Petri dish and expose it for two minutes to the fumes of a few drops of chloroform placed in the bottom of the dish. The dish must be kept tightly covered.

3. Immerse the slide in chloroform in a second dish for two minutes to complete the hardening process.

4. Transfer to carbol xylol in a Coplin jar for thirty minutes for clearing.

5. Mount in balsam in the usual way.

Celloiden of the right consistency may be made by dissolving four grams of dry celloiden in a mixture of 100cc. of absolute alcohol and 100cc. of ether. Since a celloiden mixture grows thicker in time unless it is kept absolutely air tight, it may be necessary to add alcohol and ether occasionally. Some experience is required in pouring the celloiden over the slide. One should go over the process with blank slides before attempting to coat sections. Care must be taken not to let the sections dry at any stage.

Mention may also be made of the advantage in embryology of having the serial sections of an entire chick embryo on a single slide. This facilitates not only the study of the embryo but also the issuing and storing of slides. A chick embryo as old as forty-eight hours can be put readily on a single 1″ x 3″ slide by trimming the embryo

closely and cutting the sections at least thirty microns thick. Seventy-two hour embryos can be put on single 2″ x 3″ slides conveniently by observing the same precautions. H. W. HANN, Urbana, Ill.

FAST GREEN, A SUBSTITUTE FOR LIGHT GREEN S F YELLOWISH. A sample of the Fast Green FCF, just added to the list of food dyes permitted by the government, was sent to the Stain Commission by the Warner Johnson Company for testing out as a biological stain. Fast green is a dye of the triphenylmethane group and similar to light green in composition.[1] The latter dye has been notorious for its exasperating quality of fading. Since fast green has proved more permanent than light green as a food dye, it was tried out in the technics in which light green is used in biological staining in the hope that here, too, it would prove to be a more permanent acid green dye. The safranin-light green technic[2] was selected as the most severe test; for in that technic the acid dye acts both as a differentiating agent and a counterstain. The best certified sample of light green, classed in certification tests as excellent, was used for making this comparison.

In the first test, onion root tips fixed in strong Flemming were used. They were stained in 1% aqueous safranin varying lengths of time according to the sample of safranin used, then counterstained, some in 0.2% alcoholic light green and some in 0.2% alcoholic fast green, controlling this process under the microscope. The completed slides were placed in front of a sunny window for the three summer months. At the end of this time the light green slides showed no green at all. The fast green slides were still green in all cases, and those which had been rather deeply counterstained still showed very good staining. Those which had been delicately counterstained were too pale a green.

Fast green was next tried as a counterstain with safranin on animal tissues fixed in Bouin and Zenker—intestine, kidney, spleen, skin, and developing bone. It gave a brilliant stain and good differentiation.

These tests brought out the following differences between fast green and light green: (1) Fast green is slightly bluer than light green, (2) stains more intensely in a shorter time, (3) destains safranin more slowly, thus making accurate differentiation easier, (4) is much more permanent than light green. RACHEL HAYNES, Geneva, N. Y.

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[1]Johnson, H. and Staub, P. A Proposed New Food Dye. Ind. Eng. Chem. 19, 497–8. 1927.

[2]Chamberlain, C. J. Methods in Plant Histology. *4th edition.* Chicago. 1924. See p. 61.